Altered vitamin D metabolism in type II diabetic mouse glomeruli may provide protection from diabetic nephropathy

Y Wang[1], J Zhou[1], AW Minto[1], BK Hack[1], JJ Alexander[1], M Haas[2], YC Li[1], CW Heilig[1] and RJ Quigg[1]

[1]*Department of Medicine, The University of Chicago, Chicago, Illinois, USA and* [2]*Department of Pathology, Johns Hopkins Medical Institute, Baltimore, Maryland, USA*

The *db/db* mouse develops features of type II diabetes mellitus as the result of impaired signaling through its abnormal leptin receptor. In spite of accurate metabolic features of diabetes, renal disease manifestations in these mice are not as severe as in humans suggesting the presence of protective genes. There is a growing body of evidence in humans for the relevance of vitamin D in diabetes. Here we followed a large cohort of *db/db* mice and their non-diabetic *db/+* littermates. Transcriptional profiling revealed significant upregulation of 23 genes involved in Ca^{2+} homeostasis and vitamin D metabolism in *db/db* glomeruli relative to *db/+* glomeruli. Increased glomerular expression of vitamin D_3 1α-hydroxylase, vitamin D binding protein, calbindins D9K and D28K, and calcyclin mRNA was confirmed by quantitative reverse transcription–polymerase chain reaction in 20-, 36-, and 52-week-old *db/db* glomeruli. Although vitamin D_3 1α-hydroxylase protein was primarily expressed and upregulated in *db/db* renal tubules, it was also expressed in glomerular podocytes *in vivo*. Serum 1,25-dihydroxyvitamin D_3 and urinary Ca^{2+} excretion were increased >3-fold in *db/db* mice compared to *db/+* mice. Cultured glomerular podocytes had mRNA for vitamin D_3 1α-hydroxylase, vitamin D receptor, and calbindin D28K, each of which was increased in high glucose conditions. High glucose also led to enhanced production of fibronectin and collagen IV protein, which was blocked by 1,25-dihydroxyvitamin D_3. These results show that vitamin D metabolism is altered in *db/db* mice leading to metabolic and transcriptional effects. The podocyte is affected by paracrine and potentially autocrine effects of vitamin D, which may explain why *db/db* mice are resistant to progressive diabetic nephropathy.

Kidney International (2006) **70,** 882–891. doi:10.1038/sj.ki.5001624; published online 5 July 2006

KEYWORDS: vitamin D; extracellular matrix; diabetic nephropathy; diabetes mellitus; calcium

Correspondence: *RJ Quigg, Section of Nephrology, The University of Chicago, 5841 S. Maryland Ave., MC5100, Chicago, Illinois 60637, USA. E-mail: rquigg@uchicago.edu*

Received 31 October 2005; revised 12 April 2006; accepted 18 April 2006; published online 5 July 2006

Diabetes mellitus is characterized by relative or absolute insulinopenia and hyperglycemia. Type I diabetes mellitus is due to autoimmune pancreatic β-cell destruction, whereas type II diabetes mellitus is more complex, and includes abnormalities of β-cell function and tissue resistance to insulin effects.[1,2] Although clearly a major hormonal regulator of Ca^{2+}, vitamin D has non-calcemic effects on the immune system and in β-cells to facilitate insulin production.[3–7] Related to this is the growing clinical and epidemiological evidence for a protective role of vitamin D in diabetes mellitus, and conversely, the increased risk with its deficiency.[8,9]

Diabetic nephropathy occurs in up to half of patients with diabetes mellitus and currently accounts for over 45% of new cases of end-stage renal disease carrying with it a dismal 5-year survival rate of 21%, worse than that for all forms of cancer combined.[10–12] The glomerulus is primarily involved in diabetic nephropathy, with typical histological features including glomerular basement membrane thickening and mesangial matrix expansion, with development of proteinuria reflecting impaired podocyte function.[13] At a molecular level, potential mediators behind the diabetic glomerular pathology include angiotensin II,[14] glucose transporter 1,[15] growth hormone,[16] transforming growth factor-β,[17] platelet-derived growth factor,[18] connective tissue growth factor,[19] and advanced glycation end products.[20] However, the underlying pathogenesis is still poorly understood and remains an area of active investigation.

There are a number of rodent models of types I and II diabetes mellitus.[21] Mutations in the genes that encode either leptin (*Lep*) or leptin receptor (*Lepr*) result in useful mouse models of obesity and type II diabetes mellitus.[22] The diabetes mutation (db) is due to a point mutation in *Lepr*, resulting in a stop codon in the intracellular domain of the receptor, so that its signaling capacity is curtailed.[23] Homozygous $Lepr^{db}/Lepr^{db}$ mice (referred to as *db/db*) typically have elevated plasma insulin levels by 2 weeks, and hyperglycemia and recognizable obesity by 4 weeks of age. The most severely affected background strain appears to be C57BLKs, given the ultimate depletion of β cells leading to insulinopenia and severe hyperglycemia. These mice also develop hyperfiltration, albuminuria, and mesangial

expansion,[24–26] abnormalities that parallel early human diabetic nephropathy.[21,27] Yet, *db/db* mice do not have progressive renal disease, limiting their utility as a true model of human diabetic nephropathy and prompting a major search for alternatives.[21,28] Overall, given the heterogeneity of diabetic nephropathy among humans with diabetes even with equivalent glycemic control and the relative resistance of mice to diabetic nephropathy, there are likely to be genes that confer protection to diabetic nephropathy.[29]

As one potentially powerful approach to dissect mechanisms underlying diabetic nephropathy, microarrays have recently been used to profile changes in renal gene expression in glomeruli from human kidney biopsies,[26] as well as renal tissue from mice with type I diabetes mellitus induced with streptozotocin[30,31] and in type II diabetes mellitus occurring in *db/db* mice.[32,33] These studies have identified some genes that were differentially regulated in diabetic kidneys compared with their controls. Some limitations of these studies include the necessarily limited number of glomeruli obtained from human biopsy specimens, whereas the animal studies investigated transcriptional changes from whole kidney and not glomeruli where the majority of disease is concentrated, at least in early phases of disease. Although there is a gradual increase in the number of genes included on contemporary microarrays, available studies are also limited by which genes are included for study.

In the present study, we followed a large group of *db/db* and *db/+* mice up to 60 weeks of age. To screen for genes potentially relevant in the pathophysiology of diabetic nephropathy, high-density oligonucleotide microarrays were used to profile the glomerular transcriptome from these animals. Because a significant number of genes observed to be upregulated in the glomeruli of *db/db* mice were involved in vitamin D and Ca^{2+} metabolism, follow-up studies concentrated on these genes, their protein products, and potential downstream effects, with the fundamental hypothesis that these may confer protection against progressive diabetic nephropathy.

RESULTS

Differentially expressed genes in diabetic mouse glomeruli

The goal of our study was to identify glomerular genes that were differentially expressed between *db/db* and *db/+* mice and correlate these with other features of disease in these animals using carefully matched conditions. Previous studies have shown that by 16 weeks, *db/db* mice develop structural and functional changes of diabetic nephropathy.[26] We therefore generated littermate *db/db* and *db/+* mice on the C57BLKs/J background and studied them between 16 and 60 weeks of age. As expected, *db/db* mice were hyperglycemic, and developed early albuminuria and mesangial sclerosis (Table 1); however, these did not worsen as the mice aged up to 60 weeks (e.g., at no time did urine from a *db/db* mouse contain >0.5 mg albumin/mg creatinine). In these animals, there was also no evidence for renal insufficiency, as measured by blood urea nitrogen levels.

Table 1 | Manifestations of diabetes in 24-week-old *db/db* mice

	db/+ (*n*=3)	*db/db* (*n*=3)
Glucose (mg/dl)	140.3 ± 24.0	387.3 ± 15.7*
Mesangial sclerosis	0.8 ± 0.2	1.5 ± 0.1
BUN (mg/dl)	25.2 ± 2.5	27.5 ± 0.7
Urine albumin/creatinine (mg/mg)	0.04 ± 0.002	0.29 ± 08#

Bun, blood glucose and urea nitrogen.
Studied were BUN, urinary albumin excretion normalized to creatinine, and the extent of mesangial sclerosis (scored from 0 to 4) in histological specimens.
*$P<0.05$, #$P<0.01$ versus *db/+* mice.

To screen for genes differentially expressed in glomeruli from 24-week-old *db/db* mice relative to *db/+* mice, studies were performed on glomeruli isolated from littermates in each group. Using a series of relatively stringent filtering algorithms, 222 of 12 492 candidate genes were significantly changed, of which 160 genes were upregulated and 62 downregulated in glomeruli from *db/db* mice compared to those from *db/+* mice (accessible as Supplementary Data at http://madam.bsd.uchicago.edu). Of the upregulated genes, 23 were included in the gene ontology grouping of Ca^{2+} response and regulation ($P<0.001$); in the further subgroupings of biological processes and molecular functions, each was involved in Ca^{2+} ion transport and/or binding. Table 2 shows the listing of these 23 genes, which includes genes involved in vitamin D metabolism (e.g., 1α-hydroxylase and vitamin D binding protein (VDBP)), response (e.g., tumor-associated Ca^{2+} signal transducers 1 and 2), and Ca^{2+} handling (e.g., Calbindin (CaBP) D9K, CaBP D28K, and Na^{+}/Ca^{2+} exchanger, *Slc8a1*).

To extend upon the validity of these microarray data, additional experiments were performed using quantitative reverse transcription-polymerase chain reaction (qRT-PCR) in glomerular RNA from 20-, 36-, and 52-week-old animals. As shown in Figure 1, these changes identified by microarray studies were validated by this alternative method. For all but calcyclin, there also appeared to be a magnified effect as animals aged, such that the relative expression in glomeruli was highest in 52-week-old *db/db* mice compared to age-matched *db/+* mice.

Vitamin D/Ca^{2+} physiological studies

Given the altered expression of genes relevant to normal vitamin D metabolism, additional measurements were made in *db/db* and *db/+* mice to determine whether these resulted in physiological changes. Although serum Ca^{2+} was slightly elevated in *db/db* mice, this was not statistically different from *db/+* mice (Table 3). Notably, serum 1,25-dihydroxyvitamin D_3 levels and urinary Ca^{2+} excretion were both significantly elevated in *db/db* mice by greater than threefold compared to age-matched *db/+* mice. To evaluate *db/db* mice at earlier ages, separate groups of mice ($n=7$–8) were studied, which showed that 1,25-dihydroxyvitamin D_3 levels were elevated in *db/db* mice compared to age-matched *db/+* mice by 38.5% at 6 weeks and 64.4% at 12 weeks ($P<0.001$ for each group).

Table 2 | Microarray data showing calcium-related genes that were significantly increased in diabetic mouse glomeruli

Accession number	Gene symbol	Gene title	*db/db* versus *db/+* mRNA (fold-change)	*P-value*
M55413	Gc	Group specific component (VDBP)	8.66±0.92	0.001
D26352	Calbl	CaBP D28K	6.24±0.86	0.002
AB006034	Cyp27b1	Cytochrome *P*450, 27B1 (Iα-hydroxylase)	4.33±0.84	0.027
J04953	Gsn	Gelsolin	3.95±0.35	0.002
AB02807l	Calb3	CaBP D9K	3.04±0.24	0.002
AF004666	Slc8a1	Sodium/calcium exchanger	2.99±0.32	0.006
AA839903	Myl7	Myosin, light polypeptide 7	2.76±0.32	0.012
X61800	Cebpd	C/EBP, delta	2.74±0.34	0.015
X66449	S100a6	S100 calcium binding protein A6 (calcyclin)	2.68±0.13	0.001
U05809	Tkt	Transketolase	2.18±0.34	0.049
Al001633	Anxa3	Annexin A3	2.07±0.20	0.018
M76124	Tacstd1	Tumor-associated calcium signal transducer 1	2.03±0.22	0.032
Al626942	Efhd1	EF hand domain containing 1	1.99±0.08	0.001
AV003419	Anxa1	Annexin A1	1.92±0.17	0.020
M14044	Anxa2	Annexin A2	1.90±0.12	0.006
X99921	S100a13	Sl00 calcium binding protein A13	1.82±0.10	0.005
Al563854	Tacstd2	Tumor-associated calcium signal transducer 2	1.81±0.13	0.013
L27439	Pros1	Protein S (alpha)	1.77±0.16	0.043
Al845798	Pla2g12a	Phospholipase A2, group XIIA	1.63±0.10	0.014
X04017	Sparc	SPARC	1.60±0.14	0.048
U66166	Sparcl1	SPARC-like 1	1.59±0.11	0.023
Al842649	Myl9	Myosin, light polypeptide 9	1.57±0.13	0.047
X59382	Pvalb	Parvalbumin	1.52±0.07	0.008

The relative increase of the listed genes (presented as accession number, gene symbol, and common gene name) in *db/db*-compared to littermate *db/+* glomeruli is presented in descending order of calculated change, along with the associated *P*-values.
CaBP, calbindin; C/EBP, CCAAT/enhancer binding protein; SPARC, secreted acidic cysteine-rich glycoprotein; VDBP, vitamins binding protein.

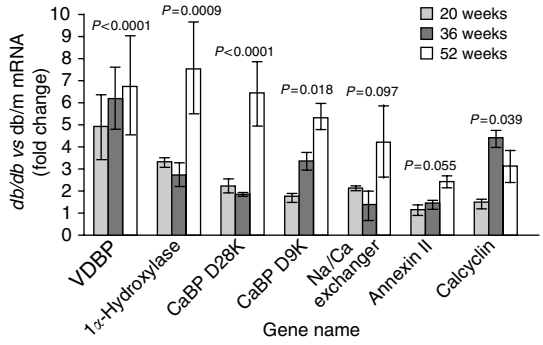

Figure 1 | Analysis of glomerular gene changes in diabetic mice over time. RNA was obtained from glomeruli isolated from *db/db* and *db/+* mice at 20, 36, and 52 weeks of age ($n = 3$ per group) followed by quantitative RT-PCR for the indicated genes. Data were normalized to 18S RNA expression from the same samples and expressed as the ratios of *db/db* to *db/+* samples. Analysis of variance was used to determine statistical differences, with generated *P*-values listed above each gene.

Although there was a marked increase in VDBP mRNA in diabetic glomeruli, the baseline mRNA levels were relatively low. Given this, and consistent with the predominant hepatic origin of this protein,[34,35] there was no difference in serum VDBP quantities between *db/db* and *db/+* mice (data not shown).

Identification of protein products in diabetic mouse kidneys

To determine the relevance of the observed transcriptional changes, kidney expression of 1α-hydroxylase and CaBP D9K and D28K proteins was determined by immunoblotting. In both glomeruli and renal cortices, all three proteins were present in *db/+* mice and were increased in *db/db* mice (Figure 2). Thus, the alterations in mRNA appear to be passed along to their translated protein products.

By immunoblotting, the relative expression of 1α-hydroxylase in the glomeruli was lower than in the renal cortex, consistent with the predominant renal tubular localization of this enzyme in humans and rodents.[36–38] To investigate this further, immunohistochemistry was performed, which showed the expected expression of 1α-hydroxylase protein in epithelia of proximal and distal tubules (Figure 3). However, 1α-hydroxylase was also present in glomerular parietal and visceral epithelial cells (podocytes, arrows in Figure 3). These findings are consistent with past studies of 1α-hydroxylase and its promoter in kidneys,[37,38] and also reflect the relatedness of epithelia of the proximal tubule and the glomerulus.

Table 3 | Calcium- and vitamin D-related physiological data from serum and urine of littermate 24-week-old *db/db* and *db/+* mice

	db/+ (*n*=61)	*db/db* (*n*=6)
1,25 dihydroxyvitamin D_3 (pg/ml)	103.8±7.3	314.7±45.9*
Calcium (mg/dl)	8.9±0.4	10.0±0.7
Phosphate (mg/dl)	7.4±1.0	8.0±1.0
Magnesium (mg/dl)	2.5±0.2	2.8±0.2
Urine calcium/creatinine (mg/mg)	0.30±0.04	1.06±0.19*

*$P < 0.05$ versus *db/+* mice.

Studies in cultured podocytes

Given the above findings showing altered mRNA and protein expression in diabetic mouse glomeruli, coupled with the

relevance of the podocyte to diabetic nephropathy, subsequent experiments were performed in cultured podocytes. Compared to low glucose conditions (5 mM), podocytes incubated in high glucose (25 mM) had increased expression of 1α-hydroxylase, CaBP 9K, and CaBP 28K (Figure 4) comparable to what was seen in *db/db* glomeruli relative to *db/+* mice (shown also in Figure 4 for the purposes of comparison).

To further confirm the presence of 1α-hydroxylase in podocytes, RT-PCR was performed to amplify full-length 1α-hydroxylase mRNA. As shown in Figure 5, renal cortex, glomeruli, and cultured podocytes all had full-length 1α-hydroxylase mRNA, which was confirmed in the latter by sequencing of the PCR product. The ability of cultured podocytes to 1α-hydroxylate 25-hydroxyvitamin D_3 was evaluated, as well as whether high glucose conditions (and induction of 1α-hydroxylase mRNA) would alter this. After 48 h exposure to 50 nM 25-hydroxyitamin D3, supernatant 1,25-dihydroxyvitamin D_3 levels were 8.6 and 19.5 pmol/l in cultured podocytes exposed to 5 and 25 mM glucose, respectively, illustrating that intrinsic podocyte 1α-hydroxylase was functional in these cells and was upregulated under high glucose conditions.

The relevance of enhanced exposure to 1,25-dihydroxyvitamn D_3, as we showed occurs *in vivo* in *db/db* mice, was explored by examining podocyte production of the key

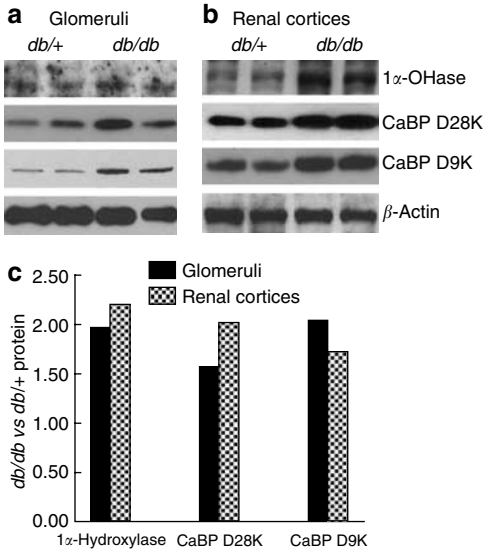

Figure 2 | Analysis of 1α-hydroxylase, CaBP D28K, and CaBP D9K proteins in diabetic mice. (**a, b**) Glomeruli and renal cortices were obtained from 24-week-old *db/db* and *db/+* mice ($n = 3$ each) and subjected to Western blotting for the individual proteins. (**c**) Studies were carried out in triplicate with representative blots shown in the figure along with data showing the relative expression of proteins in *db/db* mice compared to *db/+* mice (*C*).

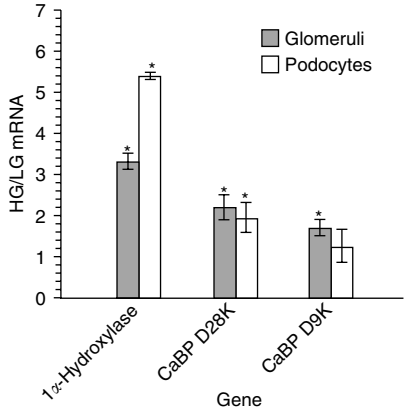

Figure 4 | Effect of high glucose on 1α-hydroxylase, CaBP D28K, and CaBP D9K mRNA in cultured mouse podocytes. Expression of the indicated genes normalized to 18S RNA from the same sample is presented as the ratio from cells cultured in high glucose (HG, 25 mM) to that from cells cultured in low glucose (LG, 5 mM) ($n = 3$, with each experiment performed in triplicate). For the purpose of comparison to data from cultured podocytes, data from 20-week-old *db/db* and *db/+* glomeruli (as shown in Figure 2) are also presented. *$P < 0.05$ high glucose versus low glucose or *db/db* versus *db/+*.

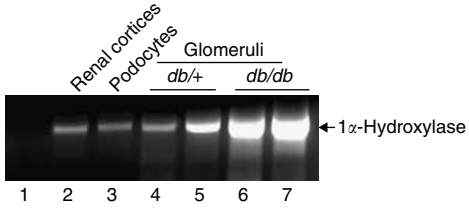

Figure 5 | Expression of full-length 1α-hydroxylase mRNA in diabetic mouse glomeruli and cultured podocytes. RNA samples from renal cortex and glomeruli from 52-week-old *db/db* and *db/+* mice and cultured podocytes were subjected to RT-PCR using primers flanking the coding sequence of 1α-hydroxylase. Controls lacking RNA were treated identically (lane 1).

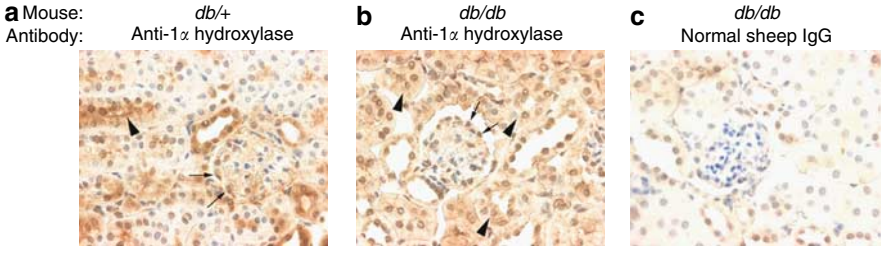

Figure 3 | Expression of 1α-hydroxylase in the kidneys of diabetic mice. Representative sections from (**a**) 36-week-old *db/+* or (**b, c**) *db/db* mice stained with (**a, b**) anti-mouse 1α-hydroxylase or (**c**) normal sheep IgG were shown. Podocytes are depicted by arrows, proximal tubular epithelium by arrowheads, and distal tubules by asterisks. (**a–c**) Original magnification × 200.

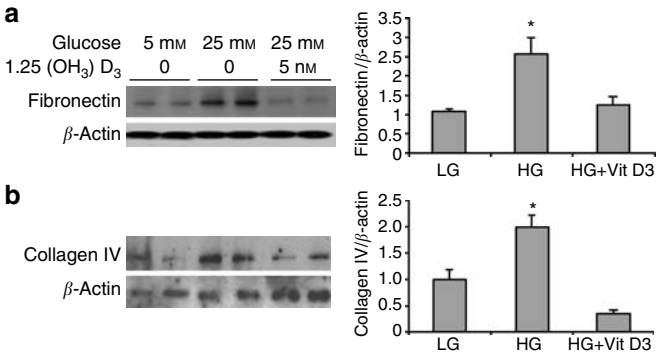

Figure 6 | Effects of high glucose and physiological 1,25-dihydroxyvitamin D_3 concentrations on (a) fibronectin and (b) collagen IV expression in cultured podocytes. Mouse podocytes were cultured low glucose (LG, 5 mM) or high glucose (HG, 25 mM) in the presence or absence of 5 nM 1,25-dihydroxyvitamin D_3. Representative Western blots for fibronectin and collagen IV with data derived by densitometry are shown ($n = 4$, with each experiment performed in triplicate). *$P < 0.02$ versus low glucose and high glucose plus 1,25-dihydroxyvitamin D_3.

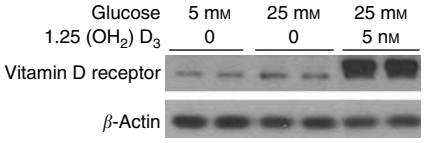

Figure 7 | Effects of high glucose and physiological 1,25-dihydroxyvitamin D_3 concentrations on VDR expression in cultured podocytes. Mouse podocytes were cultured in low glucose (5 mM) or high glucose (25 mM) in the presence or absence of 5 nM 1,25-dihydroxyvitamin D_3. A Western blot representative of eight experiments are shown.

matrix proteins, fibronectin and collagen IV. Cultured podocytes exposed to 5 mM glucose produced fibronectin (Figure 6a) and collagen IV proteins (Figure 6b), which were increased when exposed to high glucose (25 mM) conditions. Under these conditions, concomitant exposure to 5 nM 1,25-dihydroxyvitamin D_3 led to a reduction in fibronectin and collagen IV production.

Finally, given the clear importance of vitamin D receptor (VDR) to the effects of 1,25-dihydroxyvitamin D_3, its cellular expression was also assessed. Cultured podocytes in low glucose medium expressed VDR proteins of 48- and 53-kDa (Figure 7), which by analogy to human VDR are likely to be VDRA and VDRB1, respectively.[39] The quantity of the 48-kDa VDR was increased modestly (but consistently) in high glucose conditions, whereas both forms were increased further upon exposure to 1,25-dihydroxyvitamin D_3.

DISCUSSION

In this study, the transcriptional basis of diabetic nephropathy in the form exhibited in C5BLKs/J *db/db* mice was evaluated. Here we concentrated on glomeruli, where the major changes occur at least early on in the disease process. As is well known to occur, features of diabetic nephropathy were apparent in the cohort studied here, including albuminuria and mesangial matrix accumulation.[22,24] Yet, there was no evidence of progressive renal disease up to 60 weeks of age by functional or pathological criteria, which is a recognized limitation of this model as truly reflective of human diabetic nephropathy.[21,28] Given that glomeruli comprise a small fraction of total renal cortical transcripts, it is not possible to make direct comparisons to previous studies in *db/db* mice in which whole renal tissue was studied.[32,33] It is also notable that the studies by Susztak *et al.*[32] utilized proprietary cDNA arrays, whereas those by Mishra *et al.*[33] used Affymetrix oligonucleotide arrays, as we did. We therefore compared our data with those from the latter study, and identified 64 genes in common between our list of 222 genes and their 1016 genes, respectively (see Supplementary Data). Of the 23 genes in Table 2, five were in common between the two studies, which were 1α-hydroxylase, CaBP D9K, CaBP D28K, myosin light polypeptide regulatory 7, and transketolase. All five are relatively highly expressed in *db/db* glomeruli, and the former three were also documented in the whole kidney. Overall, the concordance of 28.8% (64/222) between the two studies is quite good. Besides the obvious issue of the whole kidney or isolated glomeruli being studied, there were also different approaches used in data analyses, with ours using the more contemporary robust multichip analysis approach, followed by statistical and threshold filtering.

In a short-term study in streptozotocin-induced type I diabetes in rats,[40] there was marked hypercalciuria, yet, serum 1,25-dihydroxyvitamin D_3 levels were decreased rather than increased as in our study; it is conceivable, these phenomena are related more to the toxicities of streptozotocin than diabetes.[41] The effects of leptin on 1α-hydroxylase and calcium metabolism have been shown in a recent study in *ob/ob* mice,[42] which represent a similar obese type II diabetes mellitus model as *db/db* mice. These mice had increased levels of 1α-hydroxylase and urinary calcium, which could be normalized in the short-term (48 h) by providing exogenous recombinant leptin.[42] In those studies, the acute effect of leptin on blood glucose was not reported, whereas in our studies we were unable to dissect the roles of impaired leptin signaling from those of hyperglycemia. Our ongoing experiments of transplanting normal kidneys (with normal leptin receptors) into hyperleptinemic and hyperglycemic *db/db* recipients will help address the relative roles of leptin and glucose on these findings.

It is notable that while 1α-hydroxylase is traditionally considered a proximal tubular protein,[36] contemporary studies have shown a much more widespread distribution in the kidney.[43] Furthermore, 1α-hydroxylase is also known to be expressed outside of the kidney, such as in macrophages,[44] bone cells,[45] and keratinocytes.[46] In the kidney, distal convoluted tubules and epithelia of the glomerulus have evidence for 1α-hydroxylase expression.[38] The proximal tubular 1α-hydroxylase clearly has a major endocrine role. In this system, which has now been well worked out, 25-hydroxyvitamin D_3 bound to VDBP is

filtered at the glomerulus and taken up by the proximal tubule in a megalin-dependent manner.[43,47] In the proximal tubular mitochondria, 25-hydroxyvitamin D_3 is 1α-hydroxylated and then active 1,25-dihydroxyvitamin D_3 is exported to the capillaries at the basal surface to join the systemic circulation. Enhanced renal 1α-hydroxylation of 25-hydroxyvitamin D_3 has been noted in *db/db* mice,[41] which is consistent with our findings of increased renal 1α-hydroxylase protein mass and its 1,25-dihydroxyvitamin D_3 product in plasma. In other cells, the production of 1,25-dihydroxyvitamin D_3 is believed to serve more of a paracrine and/or autocrine role.[3–5,48] This would appear to be the case also for podocytes, given the barrier to their contributing 1,25-dihydroxyvitamin D_3 to the plasma pool. It is notable that podocytes in the rat bear megalin, whereas those of humans and mice do not.[49]

The VDRs are also appreciated to have a widespread distribution with a myriad of potential effects.[3,4] Unlike many other endocrine receptors, VDRs are the product of a single gene, with little transcriptional and translational modification.[4] In humans, there are three VDR isoforms that have been recently described – the classic 48-kDa VDRA and two B isoforms, VDRB1 and VDRB2.[39] Interestingly, here we found in cultured mouse podocytes what we presume to be VDRA and VDRB1, the former of which was upregulated in high glucose conditions. VDRB1 was further increased in these cells exposed to 1,25-dihydroxyvitamin D_3 which could represent an effect on VDRB1 transcripts and/or existing VDRB1 protein.[50,51] The actions of these VDRs when bound to RXR and 1,25-dihydroxyvitamin D_3 vary considerably depending on the cell system studied.[52] These transcriptional effects certainly have relevance to diabetes mellitus, such as in cells of the immune system in autoimmune type I diabetes mellitus and also in pancreatic β-cells to downregulate major histocompatibility complex molecule expression and increase insulin release.[8,9,53] The converse that insulin, or its resistance in diabetes, has direct actions on the vitamin D system is conceivable, although apparently not a prominent effect.[41]

Podocytes are key glomerular cells by virtue of their serving as the final barrier to protein passage across the glomerular capillary wall whereas at the same time permitting the abundant glomerular ultrafiltrate to pass; as well, they contribute significantly to the production and maintenance of the glomerular basement membrane matrix.[54] Thus, podocyte pathology can have a major contribution to diabetic nephropathy, such as through a reduction in podocyte density along the glomerular basement membrane, abnormalities in the specialized slit diaphragm, and by contributing to abnormal glomerular basement membrane structure.[55–57] There is a growing body of data suggesting that 1,25-dihydroxyvitamin D_3 is protective in glomerular disease, particularly in conditions associated with excess matrix production.[58,59] A recent report has been illustrative in showing that 1,25-dihydroxyvitamin D_3 can inhibit renal interstitial myofibroblasts, an effect attributed to stimulation of hepatocyte growth factor.[60] In the rat remant kidney model, 1,25-dihydroxyvitamin D_3 protected against podocyte injury associated with their loss and hypertrophy, which was felt to explain the protection against glomerulosclerosis in this model.[61] A protective role of 1,25-dihydroxyvitamin D_3 in diabetic nephropathy could also be attributed to its negative regulation of the renin–angiotension system,[62] in keeping with the known renal benefits of blocking angiotensin II receptors in both humans and rodents with diabetes.[63,64] In the present study, our supposition that 1,25-dihydroxyvitamin D_3 was protective against diabetic nephropathy is admittedly indirect, in that elevated 1,25-dihydroxyvitamin D_3 levels were associated with the lack of progression of diabetic nephropathy in *db/db* mice. Yet, in studies with cultured podocytes, we showed that 1,25-dihydroxyvitamin D_3 inhibited the glucose-stimulated production of fibronectin and collagen IV, which may be one link to explain this phenomenon. In our preliminary studies (Sun L, Liu J, Kong J, Han M, Zhang Z, Li YC. Renal protective role of vitamin D in the development of diabetic nephropathy. *American Society of Nephrology Renal Week* 2005, Abstract TH-FC014), VDR-deficient mice with stretptozotocin-induced diabetes had much more severe disease than wild-type controls, whereas treatment with the vitamin D analogue, RO-27-5646, further limited disease in the latter animals, data in direct support of our contention here that an activated vitamin D system can be protective in diabetic nephropathy.

Besides 1α-hydroxylase, 1,25-dihydroxyvitamin D_3, and VDR, other products of potential relevance to diabetic nephropathy that were observed to be increased in *db/db* glomeruli included the calbindins, CaBP-D9K, and -D28K. These calbindins are well-known vitamin D targets;[65] thus, their upregulation may well be the result of increased 1α-hydroxylase activity in *db/db* mice. Both bind intracellular Ca^{2+} with high affinity and play key roles in vitamin D-mediated transcellular Ca^{2+} transport across intestinal and renal tubular epithelia.[66,67] CaBP-D28K has also been shown to suppress cell apoptosis,[65] which is certainly relevant in the context of diabetic nephropathy as discussed above. The expression of high levels of these CaBP in glomeruli raises new questions regarding their potential function in this site.

In summary, we have shown here that in *db/db* mice, hyperglycemia and/or impaired signaling through the leptin receptor leads to upregulated 1α-hydroxylase in the kidney with resultant increased circulating 1,25-dihydroxyvitamin D_3 levels with its attendant hormonal effects, such as hypercalcuria. As the podocytes bear VDR, its engagement by 1,25-dihydroxyvitamin D_3 from the systemic circulation, as well as that which is locally produced, has the potential to be a protective response to the metabolic derangements in diabetes mellitus. Our data support that the transcriptional effects of VDR may prevent mice from developing features evident in human diabetic nephropathy.

MATERIALS AND METHODS

Animal studies

Because of the greater severity of renal disease in *db/db* mice on the C57BLKs/J background,[21,25] this was the background strain used for

these studies. Breeding pairs heterozygous for both $Lepr^{db}$ and the misty trait (m) (original designation, C57BL/KsJ-$m+/+Lepr^{db}$; current designation, BKS.Cg-$m+/+Lepr^{db}$/J; stock number 000642) were purchased from Jackson Laboratories (Bar Harbor, ME, USA). $Db/+$ mice were interbred to generate $db/+$ and db/db mice, the latter identified by early-onset obesity and hyperglycemia, whereas the former was distinguished from mice homozygous for wild-type *Lepr* by gray coat color conferred by the misty trait ($m+/m+$). After weaning, all $db/+$ and db/db mice had free access to water and Teklad Global 18% Protein Rodent Diet 2918, containing 1.01% calcium and 38.4 ng/g (1.54 IU/g) vitamin D_3. Beginning at 20 weeks of age and extending in 4-week intervals until a final group reached 60 weeks of age, three littermate animals per group had urine and blood collected followed by euthanasia for renal tissue harvest. These studies were approved by The University of Chicago Institutional Animal Care and Use Committee.

Measurements from serum and urine

For 24-h urine collections, mice were placed in individual metabolic cages. Blood was collected by cardiac puncture. Urea nitrogen, creatinine, calcium, phosphate, and magnesium in urine and serum were detected with a Beckman Autoanalyzer (Beckman Coulter, Fullerton, CA, USA). Urinary albumin concentration was measured by a mouse albumin enzyme-linked immunosorbent assay kit (Bethyl Laboratories, Montgomery, TX, USA), and normalized to urinary creatinine. Blood glucose was measured with a glucose monitor (OneTouch SureStep, LifeScan, Inc., Milpitas, CA, USA). Measurements of 1,25-dihydroxyvitamin D_3 were made by radioimmunoassay (ImmunoDiagnostic System, Boldon, UK).

Tissue processing

Glomeruli from one kidney were isolated by injection of iron oxide particles into the renal artery followed by magnetic purification;[68] glomeruli of high purity (>98%) were confirmed by phase contrast microscopy and harvested for subsequent analyses. Renal cortex from the contralateral kidney was processed by standard techniques for protein and mRNA isolation and histological studies.

Microarray analyses of *db/db* and *db/+* glomerular mRNA

Target preparation for microarray hybridizations was based on protocols supplied by Affymetrix (Santa Clara, CA, USA) with minor modifications. Briefly, total RNA from glomeruli isolated from db/db and $db/+$ kidneys ($n=3$ for each group) was extracted using TRIZOL (Invitrogen, Carlsbad, CA, USA). The quality of all RNA samples was assured by evaluation on an Agilent Technologies 2100 Bioanalyzer (Palo Alto, CA, USA). Ten micrograms of total RNA were used to synthesize double-stranded cDNA using the SuperScript Choice System (Invitrogen). First-strand cDNA synthesis was primed with a T7-(dT_{24}) oligonucleotide. Biotin-labeled antisense cRNA was synthesized from 3 μg cDNA using a BioArray High Yield RNA Transcript Labeling Kit (Enzo Diagnostics, Farmingdale, NY, USA). After precipitation with 4.0 M lithium chloride, 20 μg cRNA was fragmented in 40 mM tris-acetate, pH 8.1, 100 mM KOAc, 30 mM MgOAc buffer for 35 min at 94°C and then hybridized with Affymetrix Mu74v2 arrays for 16 h at 60 r.p.m. and 45°C. The arrays were washed and stained with streptavidin phycoerythrin and then scanned with a GeneArray Scanner (Affymetrix).

Microarray image files were obtained through Affymetrix GeneChip software (MAS 5.0). Array quality was examined according to Affymetrix protocols and then by dChip (dChip.org) to determine the percentage of outliers on each array. Subsequent robust multichip analysis was performed. Robust multichip analysis is an R-based technique that analyzes directly from the Affymetrix microarray .cel image file and is comprised of three steps: background adjustment, quantile normalization, and summarization. In the Windows operating system, the robust multichip analysis express function was used to process the data. The outputs were $\log_2$ transformed expression index data for each probe set.

Filtering of genes was performed in the following way: first, the mean expression index data of genes in each group was calculated; differences of the mean values of each gene across the experimental and control group were calculated next. A two-tailed t-test was subsequently performed to check for potential differences between experimental and control groups. For the threshold values, as the data were $\log_2$ transformed, a difference of 1.0 would equal a twofold change. Here we used a difference of 0.6 which equals a 1.5-fold change as a threshold. For the t-test, we used 0.05 as threshold. Genes meeting both the difference and t-test criteria were selected. Gene ontology relationships and their significance were determined using the Gene Onotology function in GeneSpring software (v. 6.0, Agilent Technologies).

Studies with cultured murine podocytes

Experiments were performed with a murine glomerular visceral epithelial cell (podocyte) line kindly provided by Dr Peter Mundel (Mount Sinai Medical College, New York, NY, USA). Podocytes were first grown to ~80% confluence in collagen-coated flasks under permissive conditions for the SV40T antigen which is at 33°C in Roswell Park Memorial Institute-1640 medium with 10% fetal bovine serum and 50 U/ml γ-interferon. The cells were then subcultured in the same medium but at 37°C and without γ-interferon (nonpermissive conditions) and grown to ~50% confluence. The medium was then changed to Dulbecco's modified Eagle's medium (1.8 mM $CaCl_2$), 0.5% fetal bovine serum without γ-interferon to arrest and synchronize cell growth. Cells were then studied in γ-interferon-free Dulbecco's modified Eagle's medium containing 5% fetal bovine serum and a final glucose concentration of 5 or 25 mM. After 96 h, the medium was removed and cellular RNA was isolated for subsequent analyses. In additional experiments, vitamin D was added either as 50 nM 25-hydroxyvitamin D_3 or 5 nM 1,25-dihydroxyvitamin D_3 (Sigma-Aldrich Co., St Louis, MO, USA) which was replenished 24 h later. After 48 h, the culture medium was removed and cellular proteins isolated for immunoblotting. The measurement of 1,25-dihydroxyvitamin D_3 in the supernatant was measured by radioimmunoassay as described for serum samples; as a control (blank) for these experiments, medium containing 25-hydroxyvitamin D_3 was incubated in wells lacking cells in parallel.

Reverse transcription-polymerase chain reaction

Total RNA was isolated from cultured podocytes, glomeruli, and renal cortices using TRIZOL. The RNA was converted to cDNA using SuperScript First-Strand Synthesis System (Invitrogen). To document the presence of full-length mRNA for 25-hydroxyvitamin D_3 1α-hydroxylase (cytochrome P450, 27b1), 5′-ATATGGTCTGG CAGCTTTGG-3′ and 5′-CTGCTTCCTGGAGTTTGCTC-3′, were used to amplify its gene fragment. PCR products were verified by DNA sequencing.

For quantitative real-time RT-PCR reactions, gene-specific primers for selected genes (Table 4) were used with the SYBR

Table 4 | Primers used for RT-PCR

Gene symbol	Gene name	Forward primer	Reverse primer
M18S RNA	Mouse18S RNA	ATGGCCGTTCTTAGTTGGTG	CGCTGAGCCAGTCAGTGTAG
Gc	Group specific component	TGTTGCCTGTTTCAGCACTC	GGTGTTGGGTGTTTTTGTCC
Cyp27B1	1α-OHase	TACCTGAGCCAGGTGCTCTT	CTGCTTCCTGGAGTTTGCTC
Calb1	CaBP D28K	CCATGCTTAGGCCAGTCAGT	CTTCCGCCAGATAGAAGCAG
Calb3	CaBP D9K	CACCTGCTGTTCCTGTCTGA	TCGCCATTCTTATCCAGCTC
Anxa2	Annexin A2	CACCAACTTCGATGCTGAGA	CAAAATCACCGTCTCCAGGT
S100a6	Calcyclin	AAGCTGCAGGATGCTGAAAT	CTGGATTTGACCGAGAGAGG
Slc8a1	Na/Ca exchanger	CGAGACTGTGTCGAACCTGA	TCAGGGACCACGTAAACACA

CABP, calbindin.

Green kit (Qiagen, Valencia, CA, USA) on an ABI Prism 7700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). Murine 18S RNA was used for normalization. In preliminary studies, the specificity of each primer pair was validated by DNA sequencing of each PCR product.

Histological studies

Unless stated otherwise, all procedures were performed at room temperature. Periodic acid Schiff-stained kidney sections were evaluated by a renal pathologist (MH) for the extent of mesangial sclerosis and hyperceullarity using a numeric scale of 0–4.[69] Sections of 4 μm cut from paraffin-embedded *db/db* and *db/+* kidneys were fixed with cold methyl Carnoys. After deparaffinization and hydration of tissue sections, slides were incubated with 0.3% hydrogen peroxide in phosphate-buffered saline (PBS) to block endogenous peroxidase activity. After washing with three changes of PBS, slides were heated for 11 min in a microwave in 10 mM sodium citrate, pH 6.0. The slides were blocked with 10% donkey serum in PBS followed by an avidin/biotin block (SP-2001, Vector Laboratories, Burlingame, CA, USA). After washing with PBS, the slides were incubated overnight at 4 °C with sheep anti-mouse 1α-hydroxylase (The Binding Site, San Diego, CA, USA; 1:400 in 10% donkey serum) or nonimmune sheep serum as control. With intervening PBS washing steps, the slides were incubated sequentially with biotinylated anti-sheep IgG (Sigma-Aldrich; 1:100 in 10% donkey serum), avidin–biotin enzyme reagent (1:50 in PBS) and diaminobenzidine peroxidase substrate solution (Vector Laboratories Vectastain ABC kit). The slides were washed in water and counterstained with Mayer's hematoxylin. In all cases, the observer was masked to the origin of the slides.

Immunoblotting

Renal cortex and isolated glomeruli from *db/db* and *db/+* mice, and cultured podocytes were homogenized in radioimmunoprecipitation assay buffer (PBS including 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate and a protease inhibitor cocktail (Roche, Indianapolis, IN, USA)). The supernatant was collected after centrifugation at 13 000 r.p.m. for 30 min at 4 °C. Protein concentrations were determined with bicinchoninic acid (Pierce, Rockford, IL, USA) using bovine serum albumin as a standard. Equal amounts of proteins or volumes of sera were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electrophoretically transferred to a polyvinylidene diflouride membrane (Millipore, Bedford, MA, USA). Membranes were blocked with 5% nonfat milk for 2 h at room temperature and incubated with the following dilutions of primary antibodies: 1:500 sheep anti-mouse 1α-hydroxylase and 1:5000 rabbit anti-mouse CaBP D9K (Swant, Bellinzona, CH); 1:5000 rabbit anti-mouse CaBP D28K (Chemicon International, Inc, Temecula, CA, USA); 1:2000 rabbit anti-mouse VDBP (Biodesign International, Saco, ME, USA); 1:1000 rabbit anti-mouse VDR and 1:500 anti-mouse collagen IV (Santa Cruz Biotechnology, Santa Cruz, CA, USA); and, 1:1000 rabbit anti-mouse fibronectin and β-actin (Sigma-Aldrich). Membranes were then incubated with peroxidase-conjugated anti-sheep or rabbit IgG (1:3000 and 1:1500, respectively, Sigma-Aldrich), which were detected with the SuperSignal West Dura Chemiluminescent Kit (Pierce).

Statistical analyses

The ratio of a given mRNA to 18S RNA or protein to β-actin from the same sample measured at the same time was calculated. Owing to their nonparametric nature, data were logarithmically ($\log_e$) transformed. For comparisons over time, Bonferroni-adjusted *P*-values from an analysis of variance model that adjusted for age groups (20, 36, and 52 weeks) were derived using Stata software (v. 8, Stata Corp., College Station, TX, USA). Comparisons between two groups were made by *t*-testing.

ACKNOWLEDGMENTS

This work was supported by National Institutes of Health Grants R01DK41873 and R21DK57684 to RJQ, R01DK59327 to YCL, and U01DK60994 (Animal Models of Diabetic Complications Consortium) to CWH.

SUPPLEMENTARY MATERIAL

Supplementary data.

REFERENCES

1. DeFronzo RA, Bonadonna RC, Ferrannini E. Pathogenesis of NIDDM. A balanced overview. *Diabetes Care* 1992; **15**: 318–368.
2. Pugliese A, Eisenbarth GS. Type 1 diabetes mellitus of man: genetic susceptibility and resistance. *Adv Exp Med Biol* 2004; **552**: 170–203.
3. Dusso AS, Brown AJ, Slatopolsky E. Vitamin D. *Am J Physiol Renal Physiol* 2005; **289**: F8–F28.
4. Nagpal S, Na S, Rathnachalam R. Noncalcemic actions of vitamin D receptor ligands. *Endocr Rev* 2005; **26**: 662–687.
5. van Etten V, Mathieu C. Immunoregulation by 1,25-dihydroxyvitamin D(3): Basic concepts. *J Steroid Biochem Mol Biol* 2005; **97**: 93–101.
6. Deluca HF, Cantorna MT. Vitamin D: its role and uses in immunology. *FASEB J* 2001; **15**: 2579–2585.
7. Norman AW, Frankel JB, Heldt AM, Grodsky GM. Vitamin D deficiency inhibits pancreatic secretion of insulin. *Science* 1980; **209**: 142–145.
8. Luong K, Nguyen LT, Nguyen DN. The role of vitamin D in protecting type 1 diabetes mellitus. *Diabetes Metab Res Rev* 2005; **21**: 338–346.
9. Mathieu C, Gysemans C, Giulietti A, Bouillon R. Vitamin D and diabetes. *Diabetologia* 2005; **48**: 1247–1257.
10. Eggers PW. Medicare's end stage renal disease program. *Health Care Financ Rev* 2000; **22**: 55–60.

11. Brownlee M. A radical explanation for glucose-induced beta cell dysfunction. *J Clin Invest* 2003; **112**: 1788–1790.
12. US Renal Data System. *USRDS 2004 Annual Data Report*. National Institutes of Health, NIDDK: Bethesda, MD, 2004.
13. Jawa A, Kcomt J, Fonseca VA. Diabetic nephropathy and retinopathy. *Med Clin North Am* 2004; **88**: 1001–1036.
14. Ritz E, Orth SR. Nephropathy in patients with type 2 diabetes mellitus. *N Engl J Med* 1999; **341**: 1127–1133.
15. Heilig CW, Liu Y, England RL *et al.* D-glucose stimulates mesangial cell GLUT1 expression and basal and IGF-I-sensitive glucose uptake in rat mesangial cells: implications for diabetic nephropathy801. *Diabetes* 1997; **46**: 1030–1039.
16. Doi T, Striker LJ, Quaife C *et al.* Progressive glomerulosclerosis develops in transgenic mice chronically expressing growth hormone and growth hormone releasing factor but not in those expressing insulinlike growth factor-1. *Am J Pathol* 1988; **131**: 398–403.
17. Ziyadeh FN, Hoffman BB, Han DC *et al.* Long-term prevention of renal insufficiency, excess matrix gene expression, and glomerular mesangial matrix expansion by treatment with monoclonal antitransforming growth factor-beta antibody in db/db diabetic mice. *Proc Natl Acad Sci USA* 2000; **97**: 8015–8020.
18. Kelly DJ, Gilbert RE, Cox AJ *et al.* Aminoguanidine ameliorates overexpression of prosclerotic growth factors and collagen deposition in experimental diabetic nephropathy. *J Am Soc Nephrol* 2001; **12**: 2098–2107.
19. Riser BL, DeNichilo M, Cortes P *et al.* Regulation of connective tissue growth factor activity in cultured rat mesangial cells and its expression in experimental diabetic glomerulosclerosis. *J Am Soc Nephrol* 2000; **11**: 25–38.
20. Wendt T, Tanji N, Guo J *et al.* Glucose, glycation, and RAGE: implications for amplification of cellular dysfunction in diabetic nephropathy. *J Am Soc Nephrol* 2003; **14**: 1383–1395.
21. Breyer MD, Bottinger E, Brosius III FC *et al.* Mouse models of diabetic nephropathy. *J Am Soc Nephrol* 2005; **16**: 27–45.
22. McIntosh CHS, Pedeson RA. Noninsulin-dependent animal models of diabetes mellitus. In: McNeill JH (ed). *Experimental Models of Diabetes*. CRC Press: Boca Raton, FL, 1999, pp 337–398.
23. Chen H, Charlat O, Tartaglia LA *et al.* Evidence that the diabetes gene encodes the leptin receptor: identification of a mutation in the leptin receptor gene in db/db mice. *Cell* 1996; **84**: 491–495.
24. Like AA, Lavine RL, Poffenbarger PL, Chick WL. Studies in the diabetic mutant mouse. VI. Evolution of glomerular lesions and associated proteinuria. *Am J Pathol* 1972; **66**: 193–224.
25. Gärtner K. Glomerular hyperfiltration during the onset of diabetes mellitus in two strains of diabetic mice (C57Bl/6J db/db and C57Bl/KSJ db/db). *Diabetologia* 1978; **15**: 59–63.
26. Sharma K, McCue P, Dunn SR. Diabetic kidney disease in the db/db mouse. *Am J Physiol Renal Physiol* 2003; **284**: F1138–F1144.
27. Osterby R, Gall MA, Schmitz A *et al.* Glomerular structure and function in proteinuric type 2 (non-insulin-dependent) diabetic patients. *Diabetologia* 1993; **36**: 1064–1070.
28. Breyer MD, Bottinger E, Brosius FC *et al.* Diabetic nephropathy: of mice and men. *Adv Chronic Kidney Dis* 2005; **12**: 128–145.
29. Ng DP, Krolewski AS. Molecular genetic approaches for studying the etiology of diabetic nephropathy. *Curr Mol Med* 2005; **5**: 509–525.
30. Wada J, Makino H, Kanwar YS. Gene expression and identification of gene therapy targets in diabetic nephropathy. *Kidney Int* 2002; **61**: 73–78.
31. Wada J, Zhang H, Tsuchiyama Y *et al.* Gene expression profile in streptozotocin-induced diabetic mice kidneys undergoing glomerulosclerosis. *Kidney Int* 2001; **59**: 1363–1373.
32. Susztak K, Bottinger E, Novetsky A *et al.* Molecular profiling of diabetic mouse kidney reveals novel genes linked to glomerular disease. *Diabetes* 2004; **53**: 784–794.
33. Mishra R, Emancipator SN, Miller C *et al.* Adipose differentiation-related protein and regulators of lipid homeostasis identified by gene expression profiling in the murine db/db diabetic kidney. *Am J Physiol Renal Physiol* 2004; **286**: F913–F921.
34. White P, Cooke N. The multifunctional properties and characteristics of vitamin D-binding protein. *Trends Endocrinol Metab* 2000; **11**: 320–327.
35. Song YH, Ray K, Liebhaber SA, Cooke NE. Vitamin D-binding protein gene transcription is regulated by the relative abundance of hepatocyte nuclear factors 1alpha and 1beta. *J Biol Chem* 1998; **273**: 28408–28418.
36. Kawashima H, Torikai S, Kurokawa K. Localization of 25-hydroxyvitamin D3 1 alpha-hydroxylase and 24-hydroxylase along the rat nephron. *Proc Natl Acad Sci USA* 1981; **78**: 1199–1203.
37. Hendrix I, Anderson PH, Omdahl JL *et al.* Response of the 5′-flanking region of the human 25-hydroxyvitamin D 1alpha-hydroxylase gene to physiological stimuli using a transgenic mouse model. *J Mol Endocrinol* 2005; **34**: 237–245.
38. Zehnder D, Bland R, Walker EA *et al.* Expression of 25-hydroxyvitamin D3-1alpha-hydroxylase in the human kidney. *J Am Soc Nephrol* 1999; **10**: 2465–2473.
39. Sunn KL, Cock TA, Crofts LA *et al.* Novel N-terminal variant of human VDR. *Mol Endocrinol* 2001; **15**: 1599–1609.
40. Ward DT, Yau SK, Mee AP *et al.* Functional, molecular, and biochemical characterization of streptozotocin-induced diabetes. *J Am Soc Nephrol* 2001; **12**: 779–790.
41. Ishida H, Cunningham NS, Henry HL, Norman AW. The number of 1,25-dihydroxyvitamin D3 receptors is decreased in both intestine and kidney of genetically diabetic db/db mice. *Endocrinology* 1988; **122**: 2436–2443.
42. Matsunuma A, Kawane T, Maeda T *et al.* Leptin corrects increased gene expression of renal 25-hydroxyvitamin D3-1 alpha-hydroxylase and -24-hydroxylase in leptin-deficient, ob/ob mice. *Endocrinology* 2004; **145**: 1367–1375.
43. Bland R, Zehnder D, Hewison M. Expression of 25-hydroxyvitamin D3-1alpha-hydroxylase along the nephron: new insights into renal vitamin D metabolism. *Curr Opin Nephrol Hypertens* 2000; **9**: 17–22.
44. Adams JS, Gacad MA. Characterization of 1-alpha hydroxylation of vitamin D_3 sterols by cultured alveolar macrophages from patients with sarcoidosis. *J Exp Med* 2005; **161**: 755–765.
45. Howard GA, Turner RT, Sherrard DJ, Baylink DJ. Human bone cells in culture metabolize 25-hydroxyvitamin D3 to 1,25-dihydroxyvitamin D3 and 24,25-dihydroxyvitamin D3. *J Biol Chem* 1981; **256**: 7738–7740.
46. Bikle DD, Nemanic MK, Gee E, Elias P. 1,25-Dihydroxyvitamin D3 production by human keratinocytes. Kinetics and regulation. *J Clin Invest* 1986; **78**: 557–566.
47. Nykjaer A, Dragun D, Walther D *et al.* An endocytic pathway essential for renal uptake and activation of the steroid 25-(OH) vitamin D3. *Cell* 1999; **96**: 507–515.
48. Hewison M, Zehnder D, Bland R, Stewart PM. 1alpha-hydroxylase and the action of vitamin D. *J Mol Endocrinol* 2000; **25**: 141–148.
49. Lundgren S, Carling T, Hjalm G *et al.* Tissue distribution of human gp330/megalin, a putative Ca(2+)-sensing protein. *J Histochem Cytochem* 1997; **45**: 383–392.
50. Arbour NC, Prahl JM, Deluca HF. Stabilization of the vitamin D receptor in rat osteosarcoma cells through the action of 1,25-dihydroxyvitamin D3. *Mol Endocrinol* 1993; **7**: 1307–1312.
51. Healy KD, Frahm MA, Deluca HF. 1,25-dihydroxyvitamin D3 up-regulates the renal vitamin D receptor through indirect gene activation and receptor stabilization. *Arch Biochem Biophys* 2005; **433**: 466–473.
52. Esteban LM, Fong C, Amr D *et al.* Promoter-, cell-, and ligand-specific transactivation responses of the VDRB1 isoform. *Biochem Biophys Res Commun* 2005; **334**: 9–15.
53. Mathieu C, Badenhoop K. Vitamin D and type 1 diabetes mellitus: state of the art. *Trends Endocrinol Metab* 2005; **16**: 261–266.
54. Pavenstadt H, Kriz W, Kretzler M. Cell biology of the glomerular podocyte. *Physiol Rev* 2003; **83**: 253–307.
55. Marshall SM. The podocyte: a major player in the development of diabetic nephropathy? *Horm Metab Res* 2005; **37**(Suppl 1): 9–16.
56. Wolf G, Chen S, Ziyadeh FN. From the periphery of the glomerular capillary wall toward the center of disease: podocyte injury comes of age in diabetic nephropathy. *Diabetes* 2005; **54**: 1626–1634.
57. Dalla VM, Masiero A, Roiter AM *et al.* Is podocyte injury relevant in diabetic nephropathy? Studies in patients with type 2 diabetes. *Diabetes* 2003; **52**: 1031–1035.
58. Panichi V, Migliori M, Taccola D *et al.* Effects of 1,25(OH)2D3 in experimental mesangial proliferative nephritis in rats. *Kidney Int* 2001; **60**: 87–95.
59. Makibayashi K, Tatematsu M, Hirata M *et al.* A vitamin D analog ameliorates glomerular injury on rat glomerulonephritis. *Am J Pathol* 2001; **158**: 1733–1741.
60. Li Y, Spataro BC, Yang J *et al.* 1,25-dihydroxyvitamin D inhibits renal interstitial myofibroblast activation by inducing hepatocyte growth factor expression. *Kidney Int* 2005; **68**: 1500–1510.
61. Kuhlmann A, Haas CS, Gross ML *et al.* 1,25-Dihydroxyvitamin D3 decreases podocyte loss and podocyte hypertrophy in the subtotally nephrectomized rat. *Am J Physiol Renal Physiol* 2004; **286**: F526–F533.
62. Li YC, Kong J, Wei M *et al.* 1,25-Dihydroxyvitamin D(3) is a negative endocrine regulator of the renin–angiotensin system. *J Clin Invest* 2002; **110**: 229–238.
63. Nagai Y, Yao L, Kobori H *et al.* Temporary angiotensin II blockade at the prediabetic stage attenuates the development of renal injury in type 2 diabetic rats. *J Am Soc Nephrol* 2005; **16**: 703–711.

64. Vidotti DB, Casarini DE, Cristovam PC *et al.* High glucose concentration stimulates intracellular renin activity and angiotensin II generation in rat mesangial cells. *Am J Physiol Renal Physiol* 2004; **286**: F1039–F1045.
65. Christakos S, Liu Y. Biological actions and mechanism of action of calbindin in the process of apoptosis. *J Steroid Biochem Mol Biol* 2004; **89-90**: 401–404.
66. Li YC, Bolt MJ, Cao LP, Sitrin MD. Effects of vitamin D receptor inactivation on the expression of calbindins and calcium metabolism. *Am J Physiol Endocrinol Metab* 2001; **281**: E558–E564.
67. Zheng W, Xie Y, Li G *et al.* Critical role of calbindin-D28k in calcium homeostasis revealed by mice lacking both vitamin D receptor and calbindin-D28k. *J Biol Chem* 2004; **279**: 52406–52413.
68. Ren G, Doshi M, Hack BK *et al.* Isolation and characterization of a novel rat factor H-related protein that is up-regulated in glomeruli under complement attack. *J Biol Chem* 2002; **277**: 48351–48358.
69. Doi T, Hattori M, Agodoa LY *et al.* Glomerular lesions in nonobese diabetic mouse: before and after the onset of hyperglycemia. *Lab Invest* 1990; **63**: 204–212.